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Thermostable DNA-Polymerase from the Thermophilic Archaeon Microorganism *Archaeoglobus fulgidus* VC16 and Its Features

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Abstract—A gene (No. AF0497 GenBank, USA) was cloned from the archaeon *Archaeoglobus fulgidus* strain found in the water of hot springs. This gene contains an open reading frame of 2346 base pairs which encodes a thermostable DNA-polymerase (762 amino acid residues). A recombinant protein *Afu*-pol with molecular weight of 94 kD was isolated in an *Escherichia coli* strain used as a producer and characterized. By site-directed mutagenesis in the *afu-pol* gene the amino acid residue Glu170 was replaced with Ala; this resulted in a complete loss of the 3′-5′-exonuclease activity of the enzyme. Thus, the Glu170 residue was suggested to be directly involved in formation of the 3′-5′-exonuclease site. Physicochemical features of the exodeficient enzyme form were studied, and the possible use of *Afu*(exo⁻)-pol in the polymerase chain reaction is shown.

Key words: thermostable DNA-polymerase, *A. fulgidus*, DNA amplification, gene cloning, gene expression

DNA-polymerases are key enzymes during replication and reparation in all living organisms. They became of interest more than 40 years ago after the finding of DNA-polymerase I from *Escherichia coli*. Now more than 50 genes encoding various DNA-polymerases are known, and many of them have been isolated and characterized. These enzymes are classified based on comparison of the primary structures. DNA-polymerases are subdivided into four families [1]. The A family includes DNA-polymerases homologous to the product of the *pol*A gene which encodes DNA-polymerase I from *E. coli*. The B family includes DNA-polymerases homologous to the product of the *pol*B gene which encodes DNA-polymerase II from *E. coli*. The C family includes DNA-polymerases homologous to the product of the *pol*C gene which encodes DNA-polymerase III (the α-subunit) of *E. coli*.

Recently there has been increasing interest in studies on functions and structure of thermostable DNA-polymerases involved in the metabolism of DNA at temperatures near the melting point of DNA. The enzymes *Taq*-pol and *Tgo*-pol from the A and B family are best studied. For *Taq*-pol the crystal structure is known of the enzyme with a substrate in the active site [2], for the *Tgo*-pol the crystal structure is known of the native enzyme [3].

It should also be noted that DNA-polymerases isolated from thermophilic microorganisms *Thermus aquaticus*, *Thermus thermophilus* HB8, *Pyrococcus furiosus*, *Methanococcus jannachii*, *Pyrococcus sp. GB-D*, *Thermus litoralis*, *Thermus fumicolans*, and *Pyrococcus sp. KOD1* are widely used in such modern techniques as polymerase chain reaction (PCR) and enzymatic sequencing [4].

Studies on the structure and mechanisms of the catalytic activity of new thermostable DNA-polymerases from various sources (especially of DNA-polymerases from thermophilic archaebacteria) presenting microorganisms with high temperature growth optimums (more than 70°C) is an important and urgent problem for both science and practice.

Moreover, DNA-polymerases are interesting with respect to organization of the replication system of archaebacteria which are convenient objects for modeling of the main mechanisms in bacteria and eucaryotes.

Complete sequencing of the genome of *Archaeoglobus fulgidus* archaebacteria using GeneSmith and CRITICA program technologies revealed the pres-

Abbreviations: *Afu*-pol) DNA-polymerase from *A. fulgidus* VC16; dNTP) deoxynucleoside triphosphate; IPTG) isopropyl β-D-thiogalactopyranoside; PMSF) phenylmethylsulfonyl fluoride.

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ence of 2436 open reading frames. The open reading frames were compared to the PDB (Protein Data Bank) protein database using the BLASTFX program, and the encoding of 1797 proteins with certain features and supposed biological roles was predicted [5].

Thus, open reading frames were determined which encoded some of the proteins responsible for DNA synthesis at high temperatures. The *Archaeoglobus fulgidus* microorganism was shown to have DNA-polymerase of the B-like DNA-polymerase family [6], and until now this DNA-polymerase had not been isolated and adequately studied.

The present work describes the first cloning and expression of the gene (No. AF0497, GenBank, USA) which encodes *Afu*-pol (762 amino acid residues) of the B-like DNA-polymerase family, the isolation and features of the recombinant protein, and the site-directed mutagenesis of the *afu-pol* gene. Possible use of DNA-polymerase from the thermostable microorganism archaeon *A. fulgidus* VC16 for the polymerase chain reaction is shown.

MATERIALS AND METHODS

The following materials were used: *A. fulgidus* VC16 strain from the collection of the Institute of Agricultural Biotechnology (IAB), Russian Academy of Agricultural Sciences; *E. coli* M15 strain; plasmid DNA-pREP4 and the pQE16 vector for expression (Quagen, Germany); genomic DNA from *A. fulgidus*; nutritional medium components: tryptone and yeast extract (Difco, USA). Materials used for chromatography were: heparin-Sepharose (CL-6B, Amersham Pharmacia Biotech AB, USA), Fractogel (EMD TMAE-650 (M), Merck, Germany), deoxyribonucleoside triphosphates (Medigen, Russia); oligonucleotides (Sintol, IAB, Russia); reagents for electrophoresis and buffers (Sigma, USA); [α-^{32}P]ATP, [γ-^{32}P]ATP with specific activity of 10 MBq/mole (Izotop, Russia). Other reagents were of specific purity (Reakhim, Russia).

The following primers were used:

5′-AGCATAATTGGATCCATGGAAAGAGTTGAGGGC-3′ (Archpol-1),

5′-TGGAAAATTCGTTCCGGAGAGTTAGCTGAAGAA-3′ (Archpol-2),

5′-CCTTTCGCGTACCGCTACC-3′ (Archpol-3),

5′-GACTGCTACGAAGCCCCCG-3′ (Archpol-4),

5′-CGATTGTGCAATGCTATCAAGCTTTGGTATGCC-3′ (Archpol-5),

5′-CAAAGCTTGATAGCATTGCACAATCGAAAACGAGC-3′ (Archpol-6),

5′-ACTCTGAAGGTTCTCACCAACTCGTTCTACGGCTA-3′ (Archpol-7),

5′-GTAGAACGAGTTGGTGAGAACCTTGAGAGTTTGTTGC-3′ (Archpol-8),

5′-CGCCAGGGTTTTCCCAGTCACGAC-3′ (M13-for).

The *E. coli* M15 strain containing the recombinant plasmid DNA with the *afu-pol* gene was grown in 1 liter of nutritional medium of the following composition: bactotryptone (10 g/liter), yeast extract (5 g/liter), and NaCl (10 g/liter) with addition of kanamycin (25 μg/ml) and ampicillin (50-100 μg/ml). The culture was grown at 37°C to the absorption value A_{550} = 0.6 optical unit per ml, and then the inducer IPTG (0.2 mM) was added. The biomass yield was ~5 g/liter.

A fragment of genomic DNA from *A. fulgidus* VC16 (0.3 μg) was amplified in 100 μl of reaction mixture (100 mM Tris-HCl, pH 8.85 (25°C), 50 mM $(NH_4)_2SO_4$, 20 mM $MgSO_4$, 250 mM KCl, primers Archpol-1 and Archpol-2 (1 μM each), 100 μM dNTP, *Pwo*-pol (20 activity units per ml)). The cycle parameters were as follows: denaturation at 93°C for 4.4 min, annealing at 55°C for 3 min, polymerization at 72°C for 1.5 min, altogether five cycles. Further cycle parameters were as follows: denaturation at 93°C for 1 min, annealing at 63°C for 1 min, polymerization at 72°C for 1.5 min, altogether thirty cycles.

The construction, isolation and restriction analysis of the recombinant plasmid DNA were performed by standard methods [7].

Mutagenesis of the *afu-pol* gene. Site-directed mutagenesis in the region of Asp168-Glu170 codons was performed as described in [8]. Structures of the primers Archpol-5, Archpol-6, Archpol-7, and Archpol-8 are presented above. The mutagenesis of the *afu-pol* gene was performed on the pAfu plasmid. In all desired mutants with the plasmid structure in the mutagenesis region corresponding to structures of the oligonucleotides used a *Hind*III restriction site had to appear. The plasmids were chosen by restriction analysis and the nucleotide sequence of the region affected during the site-directed mutagenesis was determined by sequencing according to Sanger's method. Thus, the pAla-Afu plasmid was prepared which included the *afu-pol* gene with the mutation resulting in the pointwise substitution of the amino acid residue Glu170→Ala in the Asp168-Glu170 region of the enzyme.

DNA was sequenced according to the schedule of Promega (USA) [9].

The expression of the thermostable DNA-polymerase in *E. coli* cells was determined by the corresponding activ-

ity in crude lysates. The cells were chosen from agar medium, night culture was grown at 37°C, 2 ml were taken, and the cells were precipitated by centrifugation at 14,000 rpm and lysed at 4°C in 50 μl of buffer which contained 10 mM KCl, 20 mM Tris-HCl (pH 8.8), 10 mM $(NH_4)_2SO_4$, 0.1% Triton X-100, lysozyme (10 mg/ml). The cell lysates were heated for 30 min at 75°C, and the denatured proteins were precipitated by centrifugation (14,000 rpm). The supernatant (5 μl) was added to 50 μl of the reaction mixture (10 mM KCl, 20 mM Tris-HCl, pH 8.8 at 25°C, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100, the activated DNA (2 optical units per ml), 100 μM dNTP including [α-^{32}P]ATP (2 MBq/ml). The control sample was supplemented with *Vent*-pol (2 activity units). The samples were incubated at 75°C for 60 min. Aliquots were taken 10, 20, 30, 40, and 60 min after beginning the reaction. Afterwards the work was performed as described earlier [10].

Isolation of the thermostable DNA-polymerase. *E. coli* cells (4.3 g) were suspended in 43 ml of buffer of the following composition: 50 mM Tris-HCl (pH 8.8, 25°C), 0.5% Triton X-100, 0.1 mM EDTA, 1 mM dithiothreitol (DTT), 3 mM PMSF, lysozyme (10 μg/ml). The suspension was ultrasonicated, heated at 75°C for 45 min, cooled, and centrifuged for 25 min with a JA-20 rotor (Beckman, USA) at 17,000 rpm.

The supernatant was subjected to column chromatography on CL-6B heparin-Sepharose (Amersham Pharmacia Biotech AB). The enzyme was eluted with a NaCl concentration gradient (0.05-0.75 M).

The fractions containing *Afu*-pol were combined, diluted tenfold, and placed onto a column with Fractogel EMD TMAE-650 (M). The enzyme was eluted with a NaCl concentration gradient (0.1-0.3 M).

The fractions containing *Afu*-pol were supplemented with glycerol to the concentration of 50%. The enzyme was stored at −20°C.

The purity of the preparation and the molecular weight of the isolated protein were monitored by electrophoresis in polyacrylamide gel with SDS by the Laemmli method.

Preparation of oligonucleotide (HP*). The oligonucleotide was 5′-phosphorylated by the standard method [7]. A mixture containing 1 μM oligonucleotide (HP) in 77 μl of bidistilled water, 1 MBq [γ-^{32}P]ATP, 10 μl of 10-fold kinase buffer, and 3 μl of the T4-polynucleotide kinase solution (Fermentas, Lithuania) (10-30 activity units) was incubated at 37°C for 30 min. The enzyme was removed by extraction with a phenol–chloroform mixture (1 : 1) and then with a mixture of chloroform–isoamyl alcohol (24 : 1). The oligonucleotide was precipitated with four volumes of cold ethanol and purified by electrophoresis in 10% denaturing polyacrylamide gel.

Optimal reaction conditions for *Afu*-pol were determined by changing concentrations of the reaction mixture components, pH, and temperature of the reaction. The reaction was performed at 25-95°C in 50 μl of the mixture which contained an activated DNA from calf thymus (2 optical units per ml), the enzyme (1.0 activity unit), dNTPs (100 μM each), 10 mM $(NH_4)_2SO_4$, 0.1% Triton X-100, [α-^{32}P]ATP (1 MBq), and also 20 mM Tris-HCl buffer (pH 6.5-10), $MgSO_4$ (1-20 mM), and KCl (0-300 mM). The enzyme activity was determined by the initial rate of the label incorporation into the acid-insoluble fraction [10]. The results were processed with the Microcal Origin program (USA).

Polymerase chain reaction with *Afu*-pol was performed in 50 μl of reaction mixture which contained 8 mM $MgSO_4$, 20 mM Tris-HCl (pH 7.5, 25°C), 0.1% Triton X-100, 10 mM $(NH_4)_2SO_4$, 100 μM dNTPs (each), the plasmid DNA (pAfu) with the cloned *afu-pol* gene (1 ng), oligonucleotides (0.2 μM), and 1 unit of the thermostable DNA-polymerase. The cycle parameters were as follows: denaturation at 94°C for 0.5 min, annealing at 50°C for 1 min, polymerization at 72°C for 2 min, altogether 30 cycles.

RESULTS AND DISCUSSION

Cloning and expression of the *afu-pol* gene. It was difficult to culture thermophilic anaerobes under laboratory conditions. Therefore, we studied the *Afu*-pol enzyme prepared by cloning and expression in *E. coli* cells of the gene (No. AF0497, GenBank, USA) from the *A. fulgidus* genome which contained the open reading frame of the protein.

In the first stage the theoretically predicted amino acid sequence of the protein was compared to the amino acid sequences of the known thermostable DNA-polymerases. We found 45% homology with DNA-polymerases of the B family and suggested that the initial gene could encode a polypeptide consisting of 762 amino acid residues.

A copy of the *Afu*-polymerase gene was prepared by polymerase chain reaction using the genomic DNA from *A. fulgidus* and two synthetic oligodeoxyribonucleotides, the Archpol-1 and Archpol-2 primers. The sequences of the primers corresponded to 5′- and 3′-ends of the desired gene and had the sites of restriction endonucleases *Bam*HI and *Kpn*2I for the gene incorporation into the expressing vector. The amplification products were separated by electrophoresis in 1.0% agarose gel. The PCR product which corresponded to the full-length gene (2346 b.p.) was treated with the *Bam*HI and *Kpn*2I restrictases and ligated with the vector based on pQE16 which was previously cleaved by the same enzymes.

The ligase mixture was transferred into *E. coli* M15 cells, and the colonies resistant to ampicillin and kanamycin were chosen. Then by restriction analysis of the plasmid DNA a plasmid was chosen which carried the full-length *afu-pol* gene. Introduction of the *afu-pol* gene into the pQE16 vector resulted in the pAfu plasmid.

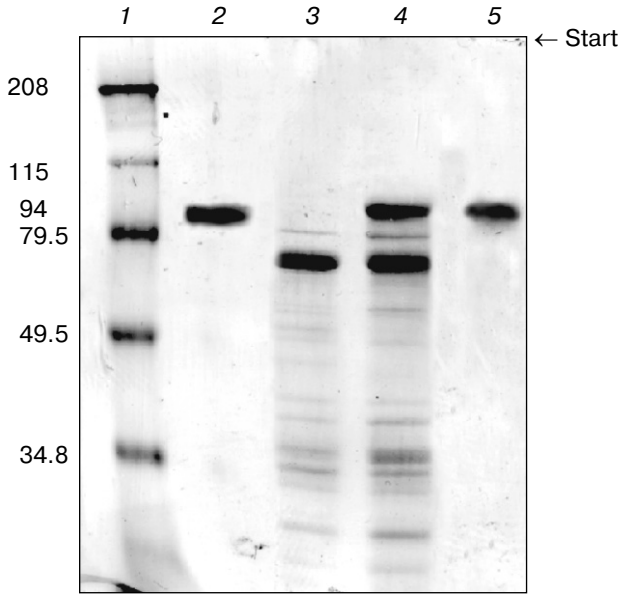

Fig. 1. Electrophoretic analysis (12% SDS-PAGE) of proteins from *E. coli* M15 cells heated at 72°C for 30 min: *1*) marker proteins, BioRad No. 72807 (myosin, β-galactosidase, BSA, ovalbumin, carbonic anhydrase); to the left their molecular weights in kD are presented; *2*) *Taq*-pol; *3*) lysate of *E. coli* M15 cells without the pAfu plasmid; *4*) lysate of *E. coli* M15 cells with the pAfu plasmid; *5*) purified *Afu*-pol.

The recombinant enzyme was purified by thermal denaturation and precipitation of the *E. coli* "host" proteins with subsequent chromatography.

The use of the producer strain allowed us to prepare a homogenous enzyme with the yield of 0.2 mg per g biomass. By SDS-PAGE electrophoresis performed as described in [11], the molecular weight of the recombinant enzyme was found to be 94 kD (Fig. 1), which corresponded to calculations.

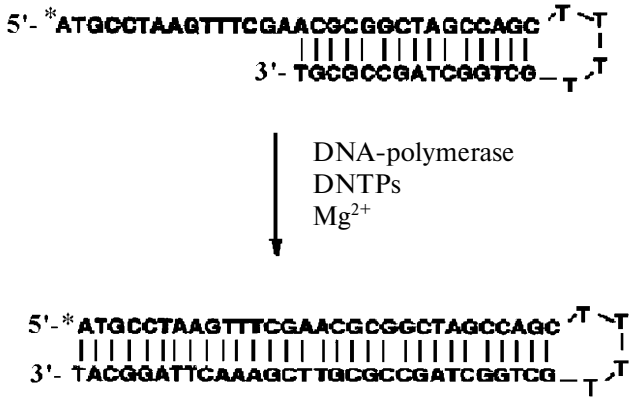

Fig. 2. Model system HP*–polymerase.

Properties of *Afu*-pol. DNA-polymerases of the B family have two enzymatic activities—the polymerase activity and 3′-5′-exonuclease activity located in different structural domains of the protein [12].

There are X-ray crystallography data on the crystal structures of DNA-polymerases of the B family (*Tgo*-pol, *Rb*69-pol) [12, 13] and on the general structure of the 3′-5′-exonuclease site. DNA-polymerases of the B family are known to have three highly conservative motifs (ExoI, ExoII, ExoIII) which form the 3′-5′-exonuclease domain and a number of conservative sequences involved in formation of the polymerase domain, e.g., KX_nNSXYG. Comparison of the *Afu*-pol primary structure to the primary structures of enzymes of the related family revealed highly conservative motifs KX_nNSXYG, ExoI, ExoII, and ExoIII in the amino acid sequence of *Afu*-pol. Therefore, this protein was suggested to have both the polymerase and 3′-5′-exonuclease activities. This hypothesis was confirmed experimentally.

Studies on the 3′-5′-exonuclease and polymerase activities of *Afu*-pol. As a model substrate synthetic oligodeoxyribonucleotide (HP*) was used which was labeled with polynucleotide kinase by the 5′-end ([γ-^{32}P]ATP) and contained the sequence capable of self-complementation at 75°C [14]. The substrate (HP*) structure is presented in Fig. 2. We found that in the presence of dNTPs *Afu*-pol could complete the substrate in the 5′→3′ direction, whereas in the absence of dNTPs it could degrade the free 3′-end of the oligodeoxyribonucleotide (Fig. 3). Consequently, the enzyme had both the polymerase and 3′-5′-exonuclease activities.

But further studies on *Afu*-pol with this model system showed that the 3′-5′-exonuclease activity compared to the polymerase activity was many times higher than in the enzymes of the family. Figure 4 presents the time dependence of HP* hydrolysis with *Vent*-pol, *Pwo*-pol, and *Pfu*-pol. *Afu*-pol could hydrolyze the substrate within the minimum time (2 min) as compared to *Vent*-pol, *Pwo*-pol, and *Pfu*-pol.

Thus, *Afu*-pol was found to have a high 3′-5′-exonuclease activity, and this made difficult further studies of the polymerase features of this enzyme. Therefore, site-directed mutagenesis of the gene was performed that allowed us to inactivate the 3′-5′-exonuclease activity.

Mutagenesis of the *afu-pol* gene. The region of *Afu*-pol for "protein design" was chosen based on the literature on the structural similarity of the 3′-5′-exonuclease site and homologies of the amino acid sequences which formed the 3′-5′-exonuclease domain of DNA-polymerases of the B family [15].

The Asp168XGlu170 region in the ExoI motif of *Afu*-pol was chosen for the mutational analysis. The amino acid residue Glu170 was to be substituted by the site-directed mutagenesis. If the Glu170 residue was

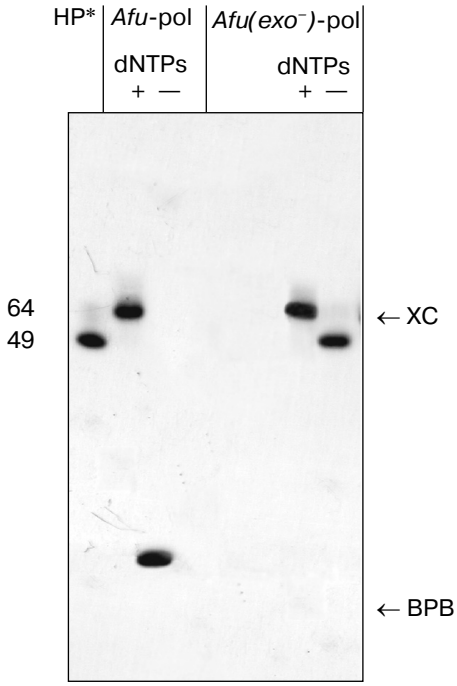

Fig. 3. Radioautograph of 10% polyacrylamide gel under denaturing conditions. Testing of the polymerase and 3′-5′-exonuclease activities of *Afu*-pol, *Afu*(exo⁻)-pol at 72°C for 30 min with the model substrate HP*. To the left, number of nucleotide bases (b.p.) in the synthetic oligodeoxyribonucleotide (HP*); the arrows show the electrophoretic mobilities of the dyes (xylene cyanole (XC), bromophenol blue (BPB)).

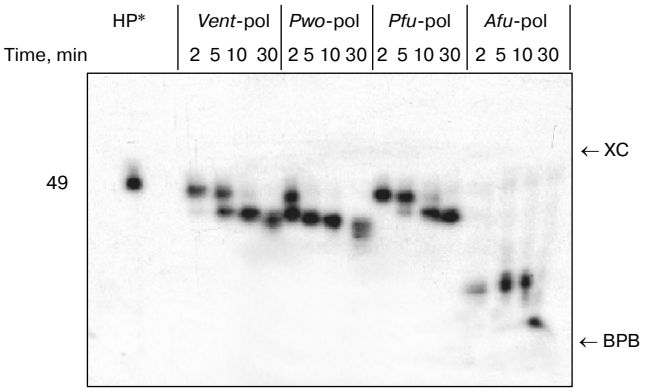

Fig. 4. Radioautograph of 10% polyacrylamide gel under denaturing conditions. Testing of the 3′-5′-exonuclease activity with the model substrate HP* in the absence of dNTPs. To the left, the number of nucleotide bases (b.p.) in the synthetic oligodeoxyribonucleotide (HP*). The arrows show the electrophoretic mobilities of the dyes (xylene cyanole (XC), bromophenol blue (BPB)).

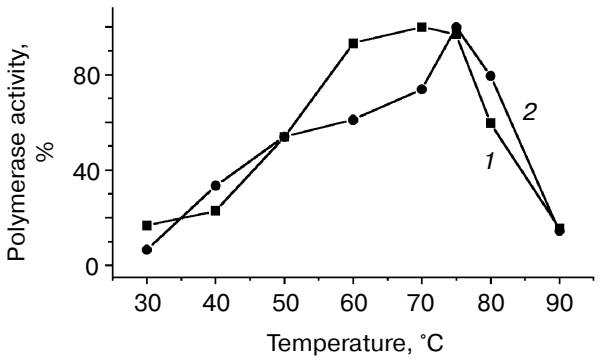

Fig. 5. Effect of temperature on the polymerase activity of *Afu*(exo⁻)-pol (*1*) and *Vent*-pol (*2*).

directly involved in formation of the 3′-5′-exonuclease site, its substitution would result in a complete loss of the 3′-5′-exonuclease activity of the enzyme, similarly to findings for *Vent*-pol, *Rb*69-pol, *DeepVent*-pol on Glu substitutions for Ala in the conservative ExoI motif [16].

The site-directed mutagenesis was performed as described in [8].

Oligodeoxyribonucleotides for the site-directed mutagenesis were constructed to provide the appearance of the restriction site *Hind*III on Glu170→Ala substitution in the region of mutagenesis. The clones which were the desired mutants were chosen by hydrolysis of the plasmids with *Hind*III endonuclease. By sequencing in the mutagenesis region the replacement of GAA (Glu170)→GCA (Ala) codons was shown. As expected, the resulting mutant form *Afu*-pol (Glu170→Ala) had no 3′-5′-exonuclease activity (Fig. 3). This mutant was called an exodeficient form of the enzyme (*Afu*(exo⁻)-pol). Subsequent studies on the polymerase features of *Afu*-pol were performed on the exodeficient form of the enzyme.

Temperature optimum. Thermostable DNA-polymerases are known to have the temperature optimum of the DNA-polymerase activity in the range of 70-75°C [17]. Microorganisms *A. fulgidus* and *T. litoralis* are closely related. Therefore, the temperature optimum of the DNA-polymerase activity of *Afu*-pol was compared to data for the well-studied *Vent*-pol from *T. litoralis*.

Figure 5 presents temperature dependences of the DNA-polymerase activities of *Afu*(exo⁻)-pol and *Vent*-pol. *Afu*(exo⁻)-pol had a broad temperature optimum (60-78°C), but it was lower than the temperature optimum of *Vent*-pol (70-80°C). These data are in good agreement with the temperature growth limits of the initial organisms: 60-95°C for *A. fulgidus* and 80-104°C for *T. litoralis* [18, 19].

To determine if *Afu*(exo⁻)-pol could be used in the polymerase chain reaction, the stability of the enzymes

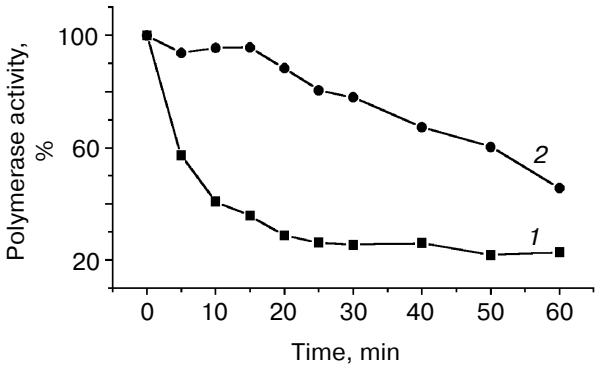

Fig. 6. Thermostability of *Afu*(exo⁻)-pol (*1*) and *Vent*-pol (*2*) at 95°C.

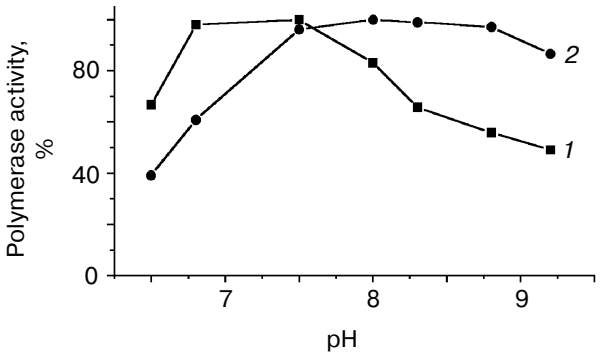

Fig. 7. Dependence of the polymerase activity of *Afu*(exo⁻)-pol (*1*) and *Pfu*-pol (*2*) on pH of the reaction mixture containing Tris-HCl buffer.

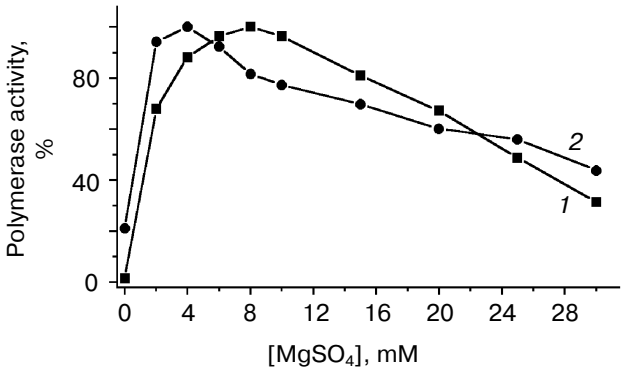

Fig. 8. Dependence of the polymerase activity of *Afu*(exo⁻)-pol (*1*) and *Pwo*-pol (*2*) on the Mg^{2+} concentration.

Afu(exo⁻)-pol and *Vent*-pol was studied at 95°C in the absence of substrates. After the enzyme heating at 95°C for a certain time the residual polymerase activity was measured under the optimal reaction conditions (Fig. 6). The *Afu*(exo⁻)-pol enzyme was found to have a half-inactivation period of about 7 min. This was somewhat lower than the value obtained for *Taq*-pol (12 min) [20]. After incubation at 95°C for 60 min *Afu*(exo⁻)-pol and *Vent*-pol retained 20 and 50% of the polymerase activity, respectively. The temperature optimum (60-78°C) and the thermostability level of *Afu*(exo⁻)-pol in the reaction mixture under the conditions described allowed this enzyme to be used for both practice and further studies on the function mechanism of thermostable DNA-polymerases.

Effect of pH on the DNA-polymerase activity. The effect of pH on the polymerase features of *Afu*(exo⁻)-pol was studied over a wide pH range from 5.5 to 9.8. To compare the pH optimum, *Pfu*-pol from *P. furiosus* with the pH optimum 8.0-8.5 was used, whereas the pH optimum for the enzyme from *A. fulgidus* was not higher than 7.5. Figure 7 presents the pH-dependence of the enzyme activity, and *Afu*(exo⁻)-pol displayed the polymerase activity optimum at pH 6.8-7.5, whereas the pH optimum for *Pfu*-pol was 7.5-8.8 (at 25°C). Thus, *Afu*(exo⁻)-pol had the optimum of the polymerase activity at lower values of pH than *Pfu*-pol, which is in line with data on the natural conditions of survival of the initial microorganisms *A. fulgidus* and *P. furiosus* [19].

The effect of bivalent ions on the polymerase features of *Afu*(exo⁻)-pol was compared to their effects on *Pwo*-pol. The cofactor of DNA-polymerases Mg^{2+} as $MgSO_4$ was taken as the metal ion. Dependences of the *Afu*-pol and *Pwo*-pol activities on the concentration of the bivalent ion Mg^{2+} are presented in Fig. 8. The *Afu*(exo⁻)-pol activity was maximal at 8 mM Mg^{2+}. At this concentration the activity of *Pwo*-pol was lower. The optimal concentration of Mg^{2+} for *Pwo*-pol was 4 mM. As compared to *Pwo*-pol, *Afu*(exo⁻)-pol displayed the maximal polymerase activity over a wider range of Mg^{2+} concentrations, from 4 to 10 mM.

Effect of univalent ions on the polymerase features of *Afu*(exo⁻)-pol. The enzyme activity was influenced by Na^+ and K^+ in the reaction mixture. Figure 9 shows a decrease in the enzyme activities with increasing KCl concentration. Thus, at 100 mM KCl the residual activity of *Afu*(exo⁻)-pol was only 2%, whereas *Pwo*-pol retained 60% of the maximal activity. Obviously, the sensitivities of *Afu*(exo⁻)-pol and *Pwo*-pol to KCl were significantly different. On replacement of KCl with NaCl the dependence of the enzyme activities on the ionic strength of the solution was not changed.

Processivity of *Afu*-pol. Processivity is an important feature of DNA-polymerases. It is defined by the number of nucleotide residues incorporated with a DNA-polymerase molecule per catalytic act into the growing 3′-end of DNA. The processivity of *Afu*(exo⁻)-pol was deter-

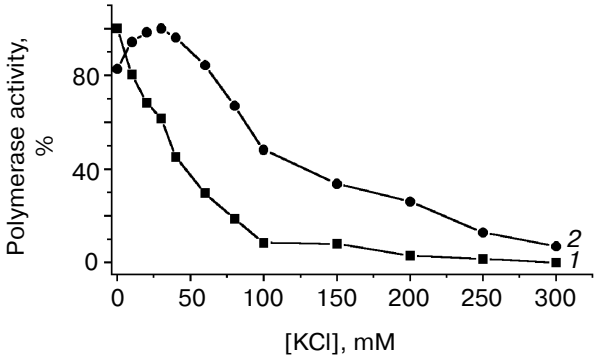

Fig. 9. Dependence of the polymerase activity of *Afu*(exo⁻)-pol (*1*) and *Pwo*-pol (*2*) on the KCl concentration.

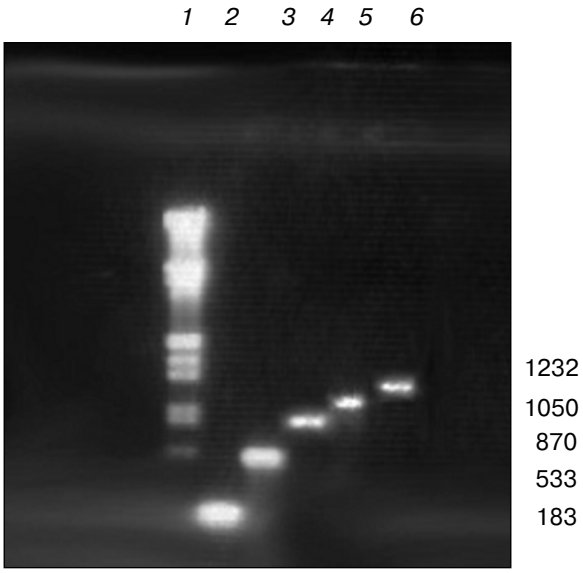

Fig. 10. Amplification of DNA fragments of 180-1300 b.p. in length: *1*) marker DNA (phage λ *Eco*RI/*Hind*III DNA); *2-5*) results of the polymerase chain reaction with *Afu*(exo⁻)-pol. To the right, the length (b.p.) of the amplification products is shown.

mined as described in [21]. A single-strand DNA of the M13 phage as a model system and a primer M13-for were taken. The DNA, primer, and the enzyme were incubated at 72°C in the presence of all nucleoside triphosphates. Aliquots were taken from the reaction mixture after 20 sec and 1, 2, and 4 min. The reaction products were analyzed by denaturing 6% PAGE. The *Afu*(exo⁻)-pol processivity was found to be ~20 b.p. that was in agreement with data for DNA-polymerases of the related family (*Vent*-pol, *Pwo*-pol, *Pfu*-pol) [17].

Amplification of DNA. The practical importance of thermostable DNA-polymerases is closely associated with their ability to amplify DNA for polymerase chain reaction. And in the next stage we attempted to use *Afu*(exo⁻)-pol for PCR. Based on physicochemical data, the reaction conditions were chosen and the buffer composition was found to promote the manifestation of the polymerase features by *Afu*(exo⁻)-pol. The buffer composition was as follows: 8 mM $MgSO_4$, 20 mM Tris-HCl (pH 7.5, 25°C), 0.1% Triton X-100, 10 mM $(NH_4)_2SO_4$, dNTPs (100 μM each). The amplification proceeded by the following schedule: denaturation at 94°C for 0.5 min, annealing at 50°C for 1 min, and polymerization at 72°C for 2 min, altogether 30 cycles.

The genomic DNA from *A. fulgidus* was taken as the template. The primers are presented in "Materials and Methods". The amplification results are shown in Fig. 10. By the polymerase chain reaction products of 180-1300 b.p. in length were prepared. Thus, the recombinant enzyme could successfully amplify DNA fragments of various lengths.

Characteristics of thermostable DNA-polymerases of the B family

Features	*Afu*-pol	*Vent*-pol	*Deep-Vent*-pol	*Pfu*-pol	*Pwo*-pol
Molecular weight, kD	94.0	90.0	90.6	90.1	90.0
Temperature optimum of polymerase activity, °C	60-78	70-80	75-80	70-80	70-80
Optimum pH (25°C) for polymerase activity	6.8-7.5	8.8	8.8	7.5-8.8	8.8
Optimal concentration of KCl for polymerase activity, mM	0	10.0	10.0	15	25.0
Optimal concentration of Mg^{2+} for polymerase activity, mM	8.0	2.0	2.0	2.0	4.0
3′-5′-exonuclease activity	+	+	+	+	+
Processivity, b.p.	<20.0	<20.0	<20.0	<20.0	<20.0

The characteristics of *Afu*(exo⁻)-pol and of thermostable DNA-polymerases of the related family are presented in the table.

Thus, the resulting protein (*Afu*-pol) was shown to have polymerase and 3′-5′-exonuclease activities. And the 3′-5′-exonuclease activity was much higher than the polymerase activity, and this is its difference from the enzymes of the related family.

The site-directed mutagenesis resulted in a mutant protein with a point substitution of the amino acid residue Glu170→Ala, and the resulting protein did not retain the 3′-5′-exonuclease activity. Therefore, the region Asp168XGlu170 was suggested to be a part of the catalytic site of the 3′-5′-exonuclease domain and Glu170 to be directly involved in its generation.

A mutant polymerase from *A. fulgidus* called *Afu*(exo⁻)-pol had the standard level of processivity and a rather high temperature optimum. These features resembled those of DNA-polymerases of the related family (*Vent*-pol, *Pwo*-pol, *Pfu*-pol). However, in some features (pH optimum of polymerase activity, optimal concentrations of uni- and bivalent cations K^+ and Mg^{2+}) *Afu*(exo⁻)-pol was significantly different from the other polymerases. In particular, the optimal concentration of Mg^{2+} for *Afu*(exo⁻)-pol was virtually fourfold higher. Because physicochemical characteristics of the enzyme are similar to those of thermostable DNA-polymerases of the related family, it can be used in various DNA-technologies (polymerase chain reaction, enzymatic sequencing of DNA).

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